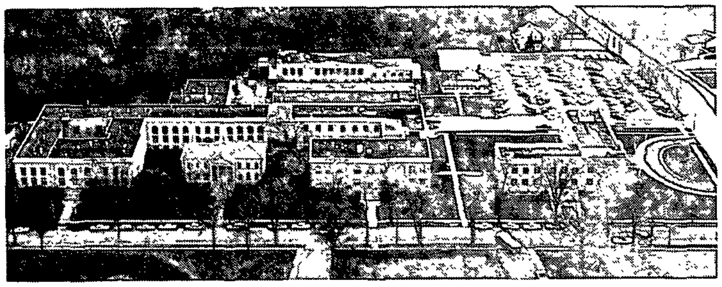

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A COMPARISON OF SELECTED WARMWATER ANIMALS AS BIOASSAY TEST ORGANISMS FOR PULP AND PAPER MILL EFFLUENTS

EUGENE F. ZANELLA, SALLY BERBEN AND MARILYN PROULX

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Eugene F. Zanella*
Group Leader

Sally Berben*
Research Assistant

Marilyn Proulx*
Research Assistant

ABSTRACT

A definitive warmwater test organism has not been universally accepted to the degree that the rainbow trout has been accepted as a cold-water test organism. Thus, a pulp mill that desires to conduct bioassays has a choice between a wide variety of fish and nonfish test organisms. In this study the relative merits of several representative warmwater organisms as bioassay animals are discussed and compared. These include the fathead minnow (*Pimephales promelas*), the bluegill (*Lepomis machrochirus*), a cladoceran (*Daphnia magna*), and a caddisfly (*Hydropsyche* sp., larval stage). Comparisons begin with information published in the open literature for these organisms. Since most published comparisons relate to pure compounds or formulations such as insecticides and herbicides, additional data were generated in the laboratory using mill effluents, simulated mill effluents, and pure compounds which might be found in some pulp and papermaking effluents. The information obtained indicates that *Daphnia* early instars are more sensitive than adults and slightly less sensitive than fathead minnows. Fathead minnows and bluegills responded identically, and the invertebrate *Hydropsyche* responded at a level comparable to *Daphnia*. The convenience, scale of operation, and sensitivity of *Daphnia* early instars greatly favor them as test organisms. It was also demonstrated that bluegills are a good substitute for fathead minnows as a bioassay test organism.

INTRODUCTION

The decision to employ a laboratory effluent bioassay to explore possible effects of an industrial effluent on biological communities is the first in a series of decisions which must be confronted by a mill. Other choices which remain include the effect to be measured (lethal or chronic), the bioassay method to be employed (static or replacement), and probably most importantly, the test organism to be used.

Except in Canada, where provincial or federal laws require testing with the rainbow trout, there are no standard or required bioassay test organisms. There are three schools of thought which govern the choice of an appropriate bioassay test organism. One is to use the most important (or sensitive) local fish (1). This approach will provide information directly relevant to a specific receiving stream or industrial facility. However, it is often difficult to obtain sufficient quantities of healthy fish of uniform size and age for locally important test species.

A second approach toward selecting a bioassay animal is to use a universal or widely accepted standard test species. A standard test species offers uniform genetic makeup and susceptibility, ready availability of healthy organisms, and a wide information base against which to evaluate results. Guppies (2) and goldfish (3) have been suggested as universal test species and, as previously mentioned, the rainbow trout is an overwhelming favorite for cold-water ecosystems. For warmwater systems, however, the fish currently recommended as a standard test species is the fathead minnow, *Pimephales promelas* (4,5).

A third strategy relevant to selecting test organisms is that of the multiparameter or multispecies approach (6), which incorporates the idea that complex ecosystem responses cannot be described using a single test organism or by the response of organisms on a single trophic level. For this reason nonfish organisms have been evaluated as test animals (7-11). The predominant nonfish test organism has been the cladoceran *Daphnia magna* (12,13).

Of the approaches described, the multiparameter assay would provide the greatest amount of information but would also require the greatest amount of effort. Thus, it is likely that a pulp or paper mill interested in performing bioassays or contracting others to do this work will have to choose a single species. Since most U.S. pulp and paper mills are on warmwater streams, the species chosen will be a warmwater species. From this point the decision becomes more difficult. The EPA document which lists recommended methods for measuring acute toxicity (14) also lists nineteen warmwater organisms or groups of organisms recommended as test species. "Standard Methods," 15th edition, does not list test organisms at all (15).

In spite of the large number of available test organisms, a few warmwater species appear to be the most likely candidates for pulp mill bioassays. These include the fathead minnow (*Pimephales promelas*), the bluegill sunfish (*Lepomis machrochirus*), the cladoceran *Daphnia magna*, and as a representative insect, larvae of the caddisfly *Hydropsyche* sp. Each of these animals has positive and negative attributes which influence its choice as a test organism, but all are animals readily available to any mill or bioassay contracting service.

The fathead minnow [which has been recommended as a standard test species (4,5)] has been widely used, and a comprehensive data base exists with respect to physiology, culture techniques, and response to many toxic compounds. The fathead is moderately hardy and has proven to be the easiest and most acceptable fish on which to do life cycle work (4-5,16). Thus it is possible to readily obtain both acute and chronic information for this species. The disadvantage is that the fathead minnow is not native to all areas of the U.S. and is subject to some supply and demand problems. It has been our experience that fatheads available in the northern midwest are wild-trapped fish which are seldom disease free and do poorly in laboratory or

*Bio-Research Group, Chemical Sciences Division, The Institute of Paper Chemistry, Appleton, Wisconsin, 54912.

mill holding facilities. On the other hand, hatcheries which culture fatheads are located in more southerly climates; fatheads from these facilities often do poorly when transferred to different water chemistries. The most dependable source of disease-free and water-acclimated fish is raising them in the laboratory, which is easily done. However, this is seldom an option for a mill wishing to do its own bioassays unless a biologist is available with full time to devote to this task.

The bluegill sunfish lacks official endorsement as a test specimen, but it is widely used. There is a fairly comprehensive data base on this species. This fish is available in any location in the U.S. where a pulp mill might be found. Thus, there is access to local wild fish, possibly from the effluent-receiving stream. The bluegill is also widely cultured as a forage fish for pond stocking and use as a food for cultured predator fish. For this reason it is easier to find locally available hatchery-raised bluegills than locally available hatchery-raised fatheads. The bluegill is a placid fish, readily tamed, and seems to adapt to laboratory holding facilities much better than fatheads.

Daphnia magna is not widely distributed in the wild and is more commonly found in hard waters in northerly regions (22). However, it is much larger than the universally distributed "wild" *Daphnia*, such as *D. pulex*, and therefore easier to work with. This organism is becoming a standard test species in its own right and has been used to assay everything from marijuana to snake venom [Viehover (17) in Scott (9)] to herbicides (11) and insecticides (12). Because *Daphnia* reproduces parthenogenetically (females produce females asexually), a genetically uniform culture can be maintained easily in a 5-gallon container in any mill laboratory or technical department. Other attributes which recommend *Daphnia* are the following:

- short life cycle (approximately 80 days at 22°C)
- small size and small test volume requirements
- ease in handling
- high fecundity
- sensitivity to large number of toxic materials
- widely researched; large data base available
- numerous animals of known age can be made available at one time
- chronic as well as acute life cycle assays can be conducted.

The larvae of the caddisfly *Hydropsyche* sp. are included in this comparison as a representative aquatic insect which may be used to expand the horizons of a toxic effects survey into other community trophic levels. This organism also merits consideration as a standard test organism or as a reference test organism. This insect larva is representative of a genus (and family) found in nearly every major stream in the U.S. that is slightly to very eutrophic. These larvae are common in all warmwater streams in the U.S. which would support a pulp mill and thereby are accessible as well as indigenous target species. They require flowing water but an air stream, paddle wheel, or water inflow arrangement is sufficient to support them. These animals are rugged enough to be handled gently but also represent one of the most sensitive insect groups in the river. Although one investigation has used caddisfly eggs as test subjects (18), caddisflies unfortunately have not been widely studied as bioassay animals.

COMPARING TEST ANIMALS

While it is desirable that a range of test subjects be assayed during a pulp mill toxicity survey, it is more likely that preliminary testing will be done with a single species. A single species test, or series of tests, will serve to determine whether there are any indications of acute toxicity and whether more extensive testing would increase the information available for a particular effluent. Unfortunately, however, the choice as to which warmwater test animal to use may not be easy to make based upon currently available information.

Published literature exists which makes comparisons between some of the test animals we have suggested for consideration. These comparisons are almost exclusively based on single toxicants rather than complex industrial effluents and seldom rely on simultaneous tests which would allow control of variables such as dilution water composition.

The most frequent comparison is between *Daphnia* sp. and "fish." One of the earliest workers to make this comparison was Anderson (13) in 1944. Anderson generated LC_{50}'s for *Daphnia magna* adults in Lake Erie water for 42 compounds which included common acids (acetic, chromic, etc.), a variety of salts, and a few alcohols. He then compared the results to listings gathered by Ellis (19) from the literature on toxicity to "fish." The comparison concludes that *Daphnia* and fish were in "fair agreement," although some discrepancies existed.

In 1970 Sanders (11) compared *Daphnia* (adults) with bluegills for 16 herbicides. These results are summarized as follows:

	x	S.D.	n	Number >100%
Daphnia 48 HR TL_{50} (%)	25.7	39.6	16	3
Bluegill 48 HR TL_{50} (%)	37.1	40.8	15	3

Under the test conditions used (normally bluegills would be exposed for 96 hours), the *Daphnia* had a lower mean TL_{50} than did the bluegill (indicating greater sensitivity). For both there were 3 herbicides which were not acutely toxic.

Buikema et al. (20) compared *Daphnia pulex* with bluegills using a composite petroleum refinery reference mixture. They found that the *Daphnia* 48-hr LC_{50} (given as multiples and fractions of the reference mixture) was 0.06, and the bluegill 96-hr LC_{50} was 5.6. Thus, the *Daphnia* was more sensitive to this compound than was the bluegill. Considerable interest has been shown in the relationship between *Daphnia* and fathead minnow chronic toxicity responses. Maki (21) tested surfactants and detergent builders and found a correlation of r = 0.98 between the responses of the two test species.

One of the most comprehensive recent comparisons of *Daphnia* and fathead minnows was done by

E. F. Kenaga (16), primarily to evaluate chronic responses. However, he included acute data for 30 related compounds and found the following:

	$\bar{x}$ LC_{50}
Daphnia	16.6 ppm
Fathead minnow	18.7 ppm

These values included some specific compounds which varied by as much as a 4-7 fold difference for both organisms. The conclusions drawn from this work are that large fish-to-fish and fish-to-*Daphnia* variability exists on a compound by compound basis, but that overall comparisons show significantly good comparisons between *Daphnia* and fathead chronic and acute toxicity responses.

One study compared *Daphnia* and fish to algae (*Chlorella*) to provide information on relative responses to toxicants (22). In this study, 49,082 chemicals were tested on *Chlorella*, with 0.54% causing 100% mortality at concentrations of 1-1.99 ppm. For *Daphnia* 2.9% of tested compounds (33,909 total) gave 100% mortality at the same concentration, and for fish 4.3% (of 35,305 compounds). The fish included fathead minnows and lake emerald shiners. The comparison concluded that *Daphnia* and fish were more sensitive than the alga *Chlorella* and that limits set to protect fish and *Daphnia* would protect algae and vascular plants.

In spite of the volume of toxicity information which exists for some of the test species available as bioassay animals, little has been published which compares responses to pulp and paper mill effluent. One exception (23) compares *Daphnia* and cold-water salmonid fish. In this study both of these organisms demonstrated a great deal of variability, but 48-hr exposures for both *Daphnia* and fish showed no differences.

EXPERIMENTAL

To expand the information available on the response of warmwater test animals to pulp and paper mill effluents, a variety of effluents and pure compounds were assayed either simultaneously or similarly. Because of the variability involved in effluent composition, comparisons of animal responses to pulp mill effluents were done simultaneously. Pure compound comparisons were made occasionally at different times but always with similar dilution water and bioassay conditions. Since much of the variability between toxicity thresholds undoubtedly stems from differences in dilution water and assay techniques (as well as test animal age and source), the greatest similarity between bioassay conditions should yield the best comparative information.

All assays used for test animal comparisons were static, no replacement. Fish and *Daphnia* assays followed the general outlines suggested by "Standard Methods" (15) and the EPA (14). Caddisfly larvae were assayed in liter jars with clock motor paddle wheels, as described in a previous report (24). Fish and caddisfly larvae were exposed for a 96-hr period, while *Daphnia* specimens were exposed for 48 hr. Both mixed age adults plus known age early instars were assayed, and comparisons were made to determine which life stage was more tolerant. Early instars were less than 24 hours old and were collected by separating gravid females from culture stocks on the day prior to testing. All *Daphnia* were obtained from a laboratory culture which is much greater than 100 generations old and which was initiated from a single female. Fathead minnows were obtained locally from wholesale bait dealers and were predominantly wild-trapped fish which varied seasonally as to age and maturity. Bluegills were obtained from a Wisconsin hatchery or flown in from a hatchery in Missouri. Caddisfly larvae were obtained from natural substrates in the Fox River in Appleton, Wisconsin. The insects were acclimated to lab water and temperatures for several weeks prior to use. Aeration of effluents and pure compounds was provided through inverted funnels or pipette tips in those cases where adequate dissolved oxygen levels could not be maintained without aeration.

Water supply for fish acclimation, *Daphnia* culture, and dilution was tap water dechlorinated with activated carbon and having a $CaCO_3$ hardness of ≈ 180. The pH was adjusted to 7 unless pH was a study variable. For pure compound assays dilution water was buffered to the desired pH using procedures recommended by the EPA (14).

Effluents tested were from a variety of mill sources located in the upper midwest and southeastern U.S., as well as a number of representative pulping and bleaching effluents generated in the laboratory in connection with other work. Pure compound comparisons were made using pentachlorophenol obtained in reagent grade from commercial sources and dehydroabietic acid (DHA) produced in the laboratory from dehydroabietonitrile obtained from Hercules, Inc. The DHA had a melting point of 169-171°C and an analyzed purity of 95-96% (gas chromatography).

Bioassay results were calculated as either LC_{50}'s or EC_{50}'s and were obtained using either the graphical interpolation method or the Litchfield Wilcoxin modified probit method (14).

RESULTS AND DISCUSSION

Daphnia Adult vs. Early Instar

Abundant data were available to use in the comparison of adult *Daphnia* to neonates less than 24 hours old. In Table 1, 48-hr EC_{50}'s are presented for paired comparisons with a variety of untreated mill effluent, untreated laboratory-generated effluent, and untreated bleach plant waste streams. For the 21 pairs of EC_{50}'s presented here it can be seen that the early instars were more sensitive than the adults in 14 of the comparisons. The early instars were less sensitive than the adults in 2 cases, and there was no difference for 5 of the effluents. Only one tested effluent was not acutely toxic (EC_{50} > 100%) to the early instars, while 4 were not acutely toxic to adult *Daphnia*. Mean values of 51.4% for adults and 35.2% for early instar *Daphnia* indicated a difference of 32% in sensitivity, with adults as the least sensitive.

The data in Table 1 were analyzed using a t-test for paired comparisons, and the differences between adults and early instars was significant at the 95% probability level [$t_{.05}$ [20] = 4.2].

Daphnia Early Instars vs. Fathead Minnows

Since it is desirable to use the most sensitive test organism or life stage when a reasonably practical choice is available, further comparisons of test animals were made using early instar daphnids.

The key comparison of *Daphnia* vs. fish was made using fathead minnows because of the fathead's growing acceptability as a warmwater standard test animal. In Table 2, fathead minnow and *Daphnia* comparisons are presented for several mill effluents as well as for pentachlorophenol (PCP) and dehydroabietic acid (DHA), which was tested at a carefully controlled pH of 6.5.

The paired data for mill effluents show that for all comparison assays the fathead minnows had a lower LC_{50} than did *Daphnia* early instars and were more sensitive. There was a 39% relative difference between the two test animals. Neither animal gave a negative acute toxicity response (LC_{50} > 100%). This difference was not statistically significant [$t_{.05}$ [4] = 2.045].

The DHA gave similar results with less difference between the test animals. *Daphnia* had a mean EC_{50} of 1.99 ppm and the fish were at 1.29 ppm. Pentachlorophenol gave similar results for *Daphnia* and fathead minnows: 0.20 ppm for *Daphnia* and 0.26 ppm for the fish. Mean values for the two sets of pure compounds showed the fatheads with the lower LC_{50}; however, this difference also was not statistically significant [$t_{.05}$ [4] = 1.187].

Fatheads Compared to Bluegills

Fewer data were available from our laboratory studies to make a comparison between bluegills and fathead minnows, but the information available (Table 3) suggests that there is no difference between the responses of bluegills and fatheads [$t_{.05}$ [3] = 0.073].

Caddisfly Larvae

The response of *Hydropsyche* to toxicants was comparable to *Daphnia* for those effluents where simultaneous comparisons were made (Table 4). *Hydropsyche* produced a mean LC_{50} of 30.4%; the *Daphnia* EC_{50} for the same effluents was 31.5%. The fathead minnow LC_{50} was lower at 25.1%, indicating greater sensitivity than either of the invertebrates; however, the differences were not large and not statistically significant [$t_{.05}$ [4] = 1.46]. For DHA, *Hydropsyche* was less sensitive than *Daphnia* but not greatly so, and the fish was more sensitive than both.

CONCLUSIONS

The literature indicates that fish and *Daphnia* are comparable with respect to overall sensitivity to a wide variety of toxic compounds. Published information also suggests that for individual compounds a very great range of responses exists for *Daphnia* as well as fish. Comparisons made at The Institute of Paper Chemistry indicate that greater consistency exists between fish and invertebrate bioassay results when simultaneous assays are conducted or when similar dilution water and procedures are used. *Daphnia* and *Hydropsyche* are slightly less sensitive than fathead minnows but not significantly so. Moreover, *Daphnia* provides the advantages of 1) a readily available and easily maintained test animal, and 2) small-scale facility requirements. These characteristics make it very useful as a mill tool. The shorter assay time and smaller facilities required should also make *Daphnia* assays less expensive as a purchased analytical test. For mills that have easier access to bluegills than to fathead minnows and prefer fish assay results, the substitution of bluegills as a test species will not affect the credibility of the results. Bluegills are also easier to raise and keep in captivity but tend to be more expensive than fatheads if obtained from a hatchery.

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Table 1. Comparison of *Daphnia magna* adult and early instars. EC_{50} as % by volume for untreated pulp and paper mill effluents

Effluent	Adult	Early Instar	% Difference
Unbleached kraft mill	70	42	40
	50	50	0
Bleached kraft mill	70	42	40
Bleached sulfite	53-8	14	74
Laboratory soda pulping	39	15	61
	24	30	-25
	33	15	54
	54	16	53
	61	40	34
	26	23	11
	26	30	-15
Laboratory sulfite bleach			
C stage	29.5	30	0
	>100	56.4	44
	>100	44.2	56
E stage	17	10	41
	5.6	4.1	26
	7.5	1.9	75
Hypochlorite	18.9	9.0	52
CEH combined	>100	>100	0
	>100	94.5	5
CED combined	94.3	74	21
Total N	21	21	
$\bar{x}$	51.4	35.2	32
S.D.	32.6	27.6	

Table 2. Comparison of *Daphnia magna* and fathead minnows. LC_{50} for unbleached pulp mill effluents and pure compound

Effluent	*Daphnia*	Fatheads	% Difference
Unbleached kraft	65%	24%	
	50	34	52
Bleached kraft	42	39	7
Bleached sulfite	14	3.4	
Kraft mill bleach plants	12	10	
	12	7.5	
Mean	32.5	19.6	39
S.D.	22.9	14.8	
Pure Compounds			
Dehydroabietic acid pH 6.5	2.47 ppm	0.89 ppm	
	1.45	1.55	
	1.95	1.45	
	2.1		
Mean	1.99	1.25	35
S.D.	0.42	0.35	
Pentachlorophenol			
	0.25	0.37	
	0.16	0.12	
		0.10	
		0.44	
Mean	0.20	0.26	-30
S.D.	0.06	0.17	
All combined pure compound data			
Mean	1.34	0.72	46
S.D.	0.93	0.57	

Table 3. Comparison of bluegill and fathead minnows response to untreated effluent and toxic compounds

	Fathead LC_{50}	Bluegill LC_{50}
Dehydroabietic acid pH 6.5 ($\bar{x}$)	1.29 ppm	4.6 ppm
Dehydroabietic acid pH 7 ($\bar{x}$)	3.2 ppm	3.2 ppm
Pentachlorophenol ($\bar{x}$)	0.24 ppm	0.19 ppm
Unbleached kraft untreated	24%	22%
Mean	7.2	7.5
S.D.	11.3	9.8

Table 4. Multiple comparisons for *Daphnia*, fathead minnow and *Hydropsyche* response to toxic compounds and untreated pulp mill effluent

Compound	*Daphnia* Early Instar EC_{50}	Fathead LC_{50}	*Hydropsyche* LC_{50}
Dehydroabietic acid pH 7 ($\overline{x}$)	7.52 ppm	3.2 ppm	10.0 ppm
Unbleached kraft	65%	24%	32%
			32
	50	34	36
Bleached kraft	42	39	35
Bleached sulfite	14	3.4	17.0
Effluent Mean	31.5	25.1	30.4
S.D.	27.3	15.7	7.7